Oil Red O and hematoxylin and eosin staining for quantification of atherosclerosis burden in mouse aorta and aortic root

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Running Head: Quantification of atherosclerosis burden in mice

Summary

Methods for staining tissues with Oil Red O and hematoxylin-eosin are classical histological techniques that are widely used to quantify atherosclerotic burden in mouse tissues because of their ease of use, reliability, and the large amount of information they provide. These stains can provide quantitative data about the impact of a genetic or environmental factor on atherosclerotic burden and on the initiation, progression or regression of the disease, and can also be used to evaluate the efficacy of drugs designed to prevent or treat atherosclerosis. This chapter provides protocols for quantifying atherosclerotic burden in mouse aorta and aortic root, including methods for dissection, Oil Red O staining, hematoxylin-eosin staining and image analysis.

Key Words: Atherosclerosis, oil red O, hematoxylin-eosin, dissection, aorta, aortic root

1- Introduction

If you are conducting a project related to atherosclerosis and plan to perform in vivo experiments with mice, it is likely that one of the questions you will try to answer is if a specific drug or a risk factor (genetic, environmental, related to behavior, etc.) has an impact on the atherosclerotic burden in the model under study (most frequently apolipoprotein E knock-out or low-density lipoprotein receptor knock-out mice) [1, 2]. An easy way to answer this question is first to detect the presence of atherosclerotic plaques and second to evaluate the size, stage and number of those plaques at different endpoints. These kinds of data will help you to conclude either that a drug or factor has no effect on the disease or, on the contrary, that it contributes to the initiation, progression, or regression of atherosclerosis. Among the methods available to accomplish this task, one of the best approaches is to use classical histological techniques such as staining with Oil Red O (ORO) and hematoxylin-eosin (H&E). These techniques not only visualize atherosclerotic lesions but also yield reliable quantitative information about the area of the aorta affected by atherosclerotic lesions, the area of each individual lesion, and the aortic intima/media ratio. Another likely concern will be where to start looking for lesions. The small size of mice makes it impractical to search in the coronary arteries as you would in humans, and it is easier to focus on the aortic root and the aorta. The more prominent lesions usually develop in the aortic arch and the arterial bifurcation, whereas lesions tend to be smaller in the descending thoracic and abdominal aorta.

In any histological staining procedure it is advantageous to know something about the chemical properties of the tissue or structure you are trying to visualize and of the stain solution being used. Visualization of lipids is challenging because they are relatively inert and, even when unsaturated, have very few sites to which stain molecules can bind. The prerequisite for any dye used to stain lipids is that it must be more soluble in the target lipid than in the vehicular solvent. The stain ORO is a fat-soluble bright red diazo dye that works simply as a pigment (an oil-soluble colorant), without forming any bond with the lipid components. ORO

effectively stains the most hydrophobic and neutral lipids in cells (such as triglycerides, diacylglycerols and cholesterol esters) but does not stain membranes because it does not stain polar lipids such as phospholipids, sphingolipids and ceramides. Analysis with ORO is moreover inexpensive and requires only basic laboratory equipment and standard computer-based software. For these reasons, ORO is widely used to identify pathological fat deposits in tissues (e.g. atheroma plaques in artery walls) or in cultured cells (abnormal accumulation of lipid droplets). ORO staining can be conducted with fresh, frozen, or formalin-fixed samples, but not with paraffin-embedded samples because the chemicals used for the deparaffinization process will also wash out most of the fats from the samples themselves. To visualize plaque lesions in paraffin sections H&E can be used.

Hematoxylin is a relatively colorless natural product that forms a functional dye when oxidized to hematein and subsequently bound to a mordant like aluminum (Al^{+3}), iron (Fe^{+3}) or chromium (Cr^{+3}). Conversion of hematoxylin to hematein used to be accomplished with the natural, spontaneous and slow action of atmospheric oxygen. To speed up the process, most current formulations incorporate a chemical oxidant. For example, the Harris formulation (the hematoxylin solution used for the protocol presented in this chapter) for many years included mercury (II) oxide as the oxidant, which has since been replaced, due to environmental concerns, with sodium iodate (Harris modified formulations). Functional hematoxylin solutions produce a blue-purple staining of cell nuclei. The specific mechanism of the staining is not totally clear, but it is believed that the color is formed through the interaction of the positively charged metal-ion–hematein complexes with the negatively charged phosphate groups in DNA. Hematoxylin can also stain acid mucins (e.g. in goblet cells of the gastrointestinal tract) and proteoglycans.

Hematoxylin solutions can be used for progressive or regressive staining. In progressive staining solutions are prepared with 1 to 4 grams of hematoxylin per liter, with the intention of staining primarily the nucleus, with a much lesser extent of coloring in the cytoplasm. With this procedure, there is no overstaining regardless of the length of staining time, and there is no need for differentiation afterwards by diluting in an acid bath. In contrast, regressive staining (often performed with the Harris formulation) requires a much more concentrated solution (5 or more grams of hematoxylin per liter) and is intended to overstain both

nuclei and cytoplasm within minutes of exposure, requiring subsequent decolorizing of the cytoplasm (differentiation) and removal of excess hematoxylin from the nucleus with dilute acid. In this procedure, omission or non-completion of differentiation will leave residual excess hematoxylin that will visually obscure fine details of nuclear chromatin and prevent the uptake of eosin. It is important to realize that hematoxylin formulations initially stain tissues a dull-looking red, and immersion in a “bluing solution” at pH5 or above is required to change the final color to bright blue. In fact, most public tap waters are sufficiently alkaline (pH5.4 to 9.8) to convert the color from red to blue (“bluing”) and are often used as a regular reagent in the procedure.

Eosin Y (Y for yellowish) is a synthetic dye derived from fluorescein and is the main component of the eosin stain, referred to in the H&E procedure as a counterstain. Due to its acidic, anionic nature, the Eosin Y molecule binds to positively charged proteins in the cytoplasm and connective tissue. Eosin thus stains nearly everything that hematoxylin does not. In addition, if used correctly, eosin staining can generate three shades of color, enabling the distinction of several tissue elements, for example red blood cells in dark reddish-orange, collagen in pastel pink, and smooth muscle in bright pink.
This chapter presents basic protocols for the dissection of mouse aorta and heart, paraffin embedding of tissues and microtome sectioning, ORO and H&E staining, and image analysis.

2-Materials

1- Mice.

2- CO_2 chamber.

3- Paper towels.

4- Cork board.

5- 25G-needles (or pins or adhesive tape).

6- Ethanol (EtOH):

6.1- Absolute (100%).

6.2- 70% EtOH in distilled water.

6-3- 80% EtOH in distilled water.

6.3- 96% EtOH in distilled water.

7- Dissection tools (*see* **Figure 1**).

8- 10-ml syringes.

9- 1.5-ml Eppendorf tubes.

10- Phosphate buffered saline (PBS): 1.54 mM KH_2PO_4, 155.17 mM NaCl, 2.70 mM Na_2HPO_4, pH 7.4.

11- Regular plastic dishes.

12- Dissecting dish (e.g. Electron Microscopy Science, Cat No 70540).

13- Microscope with camera (e.g. Olympus BX41 with Olympus UC30).

14- Fixation solution: 4% paraformaldehyde (PFA) in PBS. For 500 mL, heat 400 mL PBS in a fume hood to approximately 60°C on a hot plate with a magnetic stirrer, add 20 g of paraformaldehyde, and then add 1 M NaOH dropwise until all powder is dissolved. Make up to 500 mL with PBS, cool the solution, filter and adjust pH to 7.4. Use fresh or store frozen at -20°C.

15- Fridge (or 4ºC cold-room).

16- 0.2% ORO solution (w/v): dissolve 0.07 g ORO in 25 ml 100% methanol mixed with 10ml 1M NaOH (***see*** **Note 1**).

17- Glass funnel

18- 0.45 μm filter paper

19- Methanol:

19.1- 78% methanol in distilled water.

19.2- 100% methanol.

20- Roller and tilted mixer.

21- Histosec paraffin (paraffin enriched with polymers).

22- Oven with temperature range 1-180ºC (e.g. Memmert 100-800)

23- Paraffin embedding station.

24- Histology supplies:

24.1- Tissue processing/embedding cassettes with lids.

24.2- Pencil.

24.3- Biopsy foam pads.

24.4- Metal or plastic base molds.

24.5- Superfrost microscope slides.

24.6- Glass cover slides.

24.7- Slide storage boxes.

24.8- Slide-staining racks and jars.

24.9- Fine paint brush.

24.10- Forceps.

25- Microtome.

26- Stainless steel microtome blades.

27- Hystology paraffin bath with temperature range 0-100ºC (e.g. Termofin, PSelecta)

28- Ice bucket.

29- Xylene.

30- Fume hood.

31- Harris modified hematoxylin solution (e.g. Sigma, Ca No HHS32).

32- Eosin Y alcoholic solution (e.g. Shandon, Ca No 6766008).

33- 1% acetic acid in distilled water.

34- Xylene-based mounting medium: 45% acrylic resin and 55% xylene.

35- Stereomicroscope fitted with a digital camera and cold light (e.g. Olympus SZX10 with Olympus UC30 and Olympus KL1500 Compact).

36- Computer.

37- Software for bright-field image capture (e.g. Olympus Soft Imaging Solutions).

38- Software for image analysis and quantification (e.g. SigmaScan Pro 0.5).

3- Methods

3.1- Dissection of the mouse thoracic aorta and heart

1. Keep clean dissection tools to hand (see **Figure 1**).
2. Euthanize the mouse in a CO_2 chamber.
3. Immobilize the mouse body before dissection: with the mouse facing up, pin down (e.g. with syringe needles) the fore and hind limbs to a cork board covered with 3-4 layers of paper towels.

4. Clean and wet the mouse fur to minimize its interference: spray a small volume of 70% ethanol over the mouse abdomen.
5. Using blunt scissors and forceps, cut the mouse skin from the base of the abdomen to the top of the thorax.
6. Open the abdominal wall below the ribcage.
7. Lift the sternum with forceps and cut the diaphragm.
8. Open the ribcage bilaterally until the thymus and lungs are exposed.
9. Remove the esophagus and lungs to expose the heart and gain better access to the aorta.
10. Make 2-3 small incisions in the liver for drainage.
11. Wash out the blood from all organs using a 10 ml syringe loaded with PBS and fitted with a 25G needle: introduce the needle into the apex of the heart left ventricle and gently depress the plunger to pump 10 ml of PBS.
12. Gently dry the dissection area with paper towels.
13. Using fine scissors and forceps, grip the segment of the diaphragm that is attached to the end of the thoracic aorta, and cut the connective tissue between the aorta and thoracic cavity muscle wall to expose the aortic arch area.
14. Cut the left and right carotid arteries and the left subclavian artery to give free access to the heart and aorta (see **Figure 2A**).
15. Dissect out heart and aorta and place them in a regular plastic dish containing PBS.
16. Under a stereomicroscope, dissect the aorta by cutting at the point where it emerges from the heart.
17. Place the aorta in a 1.5-ml Eppendorf tube, add 1 ml of freshly prepared fixation solution and leave the tube at 4ºC overnight or up to 24 hours (see **Note 2**).
18. At this stage, the heart can either be processed directly for paraffin embedding **(Methods 3.4 step 1**) or placed in a tube containing 5 ml of freshly prepared fixation solution and left at 4ºC for at least 48 hours (see **Note 3**) and then processed from **step 3 in Methods 3.4.**

19. The aorta can either be paraffin embedded (**step 3 in Methods 3.4**) or processed for ORO staining (**Methods 3.2**). For ORO staining, you will need to clean the aorta by removing all adventitial fat under a stereomicroscope.

- Place the aorta in a dissecting dish, taking care to maintain it wet at all times with PBS.
- Pull away the adventitia very carefully with small forceps, avoiding excessive manipulation of the tissue.
- Cut the branches of the brachiocephalic trunk, left carotid and left subclavian arteries around 2 mm away from where they emerge.

21. Place cleaned and fixed aortas in 1.5-ml Eppendorf tubes containing PBS at 4ºC and proceed with **Method 3.2**

3.2- Staining of aorta with ORO to visualize atherosclerotic lesions

Work at room temperature.

1. Place the cleaned and fixed aortas in 1.5-ml Eppendorf tubes, one aorta per tube.
2. Add 1ml of 78% methanol to each tube and place it on a tilted roller with gentle movement for 5 minutes. Replace the methanol solution and repeat this step twice.
3. Discard the methanol and add 1ml of fresh ORO solution.
4. Incubate the tube on the tilted roller for 50 to 60 minutes (see **Note 4**).
5. Transfer the aorta to a clean tube and wash twice with 1 ml of 78% methanol for 5 minutes each on the tilted roller.
6. Discard the methanol and refill the tube with 1 ml of PBS. If necessary, at this step aortas can be stored at 4ºC.

3.3- Quantification of atherosclerotic burden in ORO-stained aortas (see Figure 3)

1. Using fine forceps, place the stained aorta on the dark surface of the dissecting dish (*see* **Note 5**).
2. Under the stereomicroscope, carefully remove any small piece of stained remnant adventitial fat that might be attached to the outer part of the adventitia.

3. Cut the aorta longitudinally: introduce the tips of a pair of microdissecting spring scissors into the artery lumen and cut the outer curvature of the aortic arch from the ascending arch to the left subclavian artery. Then continue cut along the length of the thoracic aorta.
4. Pin the aorta flat and lumen side up on the dissecting dish with steel minutien pins; insert the pins into the aorta at a small angle to minimize the shadow that they will cast on the illuminated aorta.
5. With a digital camera attached to the stereomicroscope and connected to a computer, capture a bright-field image (see **Note 6**).
6. Use appropriate software for image analysis and quantification to convert the staining in selected areas into relative quantitative data (see **Note 7**).

3.4- <u>Preparation of tissues for paraffin embedding (see Figure 4)</u>

1. For aortic root analysis, carefully trim the heart by locating the left and right atria and cutting the bottom half of the heart off with a sharp razor-blade along a plane parallel to the atria. Discard the lower half of the heart (see **Figure 2B** and **Note 8**).

2. Immerse freshly dissected aortas and trimmed hearts in fixation solution overnight at 4ºC (**Note 9**).

3. Introduce each fixed tissue sample into an individual pencil-labeled histology cassette (see **Notes 10 and 11**). Place aortic tissue between foam biopsy pads inside the cassette (see **Note 12).**

4. Immerse cassettes containing fixed tissues in 70% EtOH in a container with a lid.

5. Dehydrate tissues by immersing them in solutions with increasing concentrations of EtOH (see **Notes 13 and 14**):

30 minutes in 70% EtOH

30 minutes in 80% EtOH

30 minutes in 96% EtOH (repeat this step)

30 minutes in 100% EtOH (repeat this step)

7. In a fume hood, clear tissues by immersing the cassettes in xylene for 15 minutes (repeat this step once, changing the xylene).

8. Infiltrate the tissues with molten paraffin by immersing the cassettes in paraffin at 60ºC for 30 minutes in an oven (see **Note 15**). Discard the molten paraffin and repeat this step once.
9. Transfer the cassettes to an embedding station containing molten paraffin and incubate at 58-60ºC overnight.
10. The next day, open the cassettes, discard the non-labeled lid and choose the type of base mold (plastic or metal) that best fits the size of the sample, allowing an extra 2 mm spacing around all sides of the tissue.
11. Dispense a small amount of molten paraffin from the reservoir of the embedding station into the chosen mold.
12. Using warm forceps, transfer the tissue from the labeled-cassette lid into the base mold, taking care to position the tissue in the desired orientation.
13. Transfer the mold to a cold plate to allow solidification of a thin layer of paraffin, which will hold the tissue in place.
14. To support the tissue and ensure its identification at later steps, place the labeled cassette lid on top of the mold so that they stick and stay together (see **Note 16**).
15. Once the paraffin has cooled down and hardened to a solid, separate the paraffin block from its mold (see **Note 17**).

3.5- Microtome sectioning of paraffin-embedded aortas and hearts

1. Fill a water bath with deionized water and set it to a temperature 5º to 9ºC below the melting point of the paraffin (in preparation for the final steps of this method).

2. Cool down the paraffin blocks by placing them on the surface of melting ice for a few minutes (see **Note 18**).

3- Cut away any excess paraffin from the support cassette to facilitate secure clamping of the block in the following steps.

4. Insert the paraffin block into the specimen clamp so that the block faces the blade and is aligned in the vertical plane.

5. Before starting to trim the block, fit a new blade on the microtome (see **Note 19**) and adjust its angle to between 1º and 5º.

6. Trim the paraffin block roughly by cutting at thicknesses 10-30 µm (see **Note 20**).

7. When you arrive to the position in the block where sections begin to be of interest, change the microtome setting to 3-5-µm thickness and polish the surface of the block by gently cutting a few thin sections.

8. Using a part of the blade that has not been used for rough trimming, cut in the desired tissue plane and discard the paraffin ribbon (see **Note 21**).

9. Pick up the desired sections with forceps or a fine paint brush.

10. Flatten the sections by floating them onto the water bath with a gentle sweeping hand movement. Make sure that the smooth/shiny side of the section is facing the water (see **Note 22**).

11. Collect the sections onto the surface of clean glass slides (free of grease and dust).
12. Remove the slides from the water in a vertical movement to drain off excess water.

13. Place the slides with the paraffin sections on a warming block or in a 65°C oven for 10 to 30 minutes to allow bonding of the tissue to the glass.

14. Store ready-to-use slides at room temperature in appropriate boxes.

3.6- Hematoxylin-eosin staining of paraffin-embedded sections

1- Warm slides with paraffin-embedded section in a dry oven at 55°C for 5 to 10 minutes.

From this step on work at room temperature, in a fume hood, and use slide jars for convenience.

2- Deparaffinize the sections: fill a slide jar with xylene and immerse the slides for 5 minutes. Repeat this step twice, changing the xylene each time (see **Note 23**).

3- Rehydrate sections by passing slides through a series of decreasing concentrations of EtOH as follows (see **Note 24**):

- 2 changes of absolute EtOH, 5 minutes each.
- 2 changes of 95% EtOH, 5 minutes each.
- 2 changes of 70% EtOH, 2 minutes each.

4- Wash briefly in distilled water (see **Note 25**).

5- Stain with Harris modified hematoxylin solution for 3 minutes, monitoring the development of staining under a microscope (*see* **Note 26**).

6- Wash off excess stain with running tap water for 5 to 10 minutes.

7- Differentiate by immersing in 1% acetic acid for 30 seconds.

8- Rinse in running tap water for 5 minutes to turn the stain blue.

9- Wash in 95% EtOH for 30 seconds.

10- Counterstain in eosin alcoholic solution (diluted 1/10 in 95% EtOH) for 0.5-1 minute.

11- Wash in running tap water for 5 minutes.

12- Dehydrate sections through increasing concentrations of EtOH (see **Note 27**):

- 70% EtOH for 2 minutes.
- 95% EtOH for 2 minutes.
- Absolute EtOH for 2 minutes.

13- Clear in xylene for 2 minutes (see **Note 28** and **29**).

14- Mount with xylene based mounting medium

15- Take pictures of H&E sections with a microscope-mounted camera (see **Figure 6**)

4- NOTES

1- ORO solution can easily precipitate in contact with air. Therefore the solution, whether prepared fresh or from a stock, should always be filtered two or three times before use to remove precipitated particles that could interfere with the subsequent staining procedure. Use a glass funnel fitted with 0.45 μm filter paper

2- The fixation of the aorta facilitates the removal of adventitial fat.

3- Hearts can be fixed for longer than 48 hours without apparent damage to the tissue or creation of additional difficulties in the subsequent steps.

4- 50-60 minutes is the optimal time for staining aortic tissue with a 2% (w/v) ORO working solution. Different ORO concentrations and staining times may be required for tissues other than aorta.

5- Placing the artery on a dark surface during this step increases contrast, making manipulation of the artery under the stereomicroscope easier. A suitable dark surface can be made by fixing adhesive black tape to the silicon pad of the dissecting dish.

6- After capturing bright-field images, we improve the contrast of the image by balancing the background, using the camera software. For this we use the option “white balance” to select a blank area in the picture lying outside the tissue image itself to be used as a reference for background correction.

7- We analyze digital images of ORO-stained arteries with SigmaScan Pro 0.5, which generates an Excel sheet in which marked areas are converted to relative numbers that can be used for graphic representation of the data.

8- Before introducing a heart into a cassette, it is advisable to trim it. This will facilitate subsequent optimal embedding and sectioning, especially if you want to expose the three aortic valves in the same geometric plane.

9- If you are not going to immediately process the tissues after fixation it is better to transfer them to 70% EtOH. This step will both stop PFA fixation and advance the tissue to the first dehydration step. However, too long a time (more than 3 days) in 70% EtOH can excessively dehydrate small specimens as aortas and hearts.

10- Always write on histology cassettes with pencil; any mark with regular lab markers will be erased by the EtOH used in the dehydration process.

11- The tissues must be placed loosely in the cassette to allow enough room for ample penetration of paraffin during the embedding procedure.

12- Placing aortas between foam pads in the cassettes prevents loss of these small samples during the dehydration process and is a great help in sectioning.

13- The grade and formulation of the EtOH is important. EtOH can absorb moisture from the air, and the humidity can interfere with proper dehydration and clearing of the slides. Therefore bottles of anhydrous EtOH should be stored in lidded containers and exposure to air should be kept to a minimum.

14- Stepwise increases in EtOH concentration are used to ensure that the replacement of water in the tissue by alcohol is gradual, thereby avoiding excessive distortion of the tissue.

15- Commercial paraffin waxes used for infiltration are solid at room temperature and can be purchased with different melting points. The most common paraffin wax for histological use melts at around 56°C–58°C. In our experience it is better to use the paraffin wax at 2°C above its melting point to decrease its viscosity and improve tissue infiltration.

16- Make sure that there is enough paraffin between the mold and the cassette-lid and, if necessary, top up with hot paraffin from the paraffin dispenser. Failure to do this can lead to problems during sectioning, with unstable clamping or undesired differences in section thickness.

17- The paraffin blocks should pop out from the molds easily. If for any reason the blocks crack or the tissues are not aligned as desired inside the block, it is better to melt the blocks and start the paraffin embedding process over.

18- Sectioning is easier when the tissue inside the paraffin block and the paraffin itself are of similar hardness. For this reason, it is usually advisable to place the paraffin-embedded tissue blocks on a cold surface. In addition, if the surface is wet (e.g. melting ice), the water will penetrate slightly into the block, provoking swelling of the tissues which will make them more suitable for cutting. This is particularly important for excessively dehydrated or dry and crumbly tissues. However, placing the blocks in a freezer is not recommended because their surface will break easily.

19- Microtome blades are extremely sharp and can cause serious injury if not handled with care. Replace the microtome blade if sectioning becomes difficult.

20- The goal of trimming is to rapidly remove unwanted tissue until the region of interest is reached.

21- If the block is not ribboning well, place it back on the cool and wet surface to cool and to firm up the paraffin.

22- Flotation should expand the section to its original dimensions and leave it completely flat. If the sections break when they are placed on the water this is probably because the bath is too hot.

23- If the paraffin is not totally removed from the tissues it will interfere with subsequent staining procedures, decreasing color intensity or producing an irregular color distribution within the section

24- If the xylene from the deparaffinization step is not completely washed out by the first absolute EtOH step, subsequent carrying over of xylene into the lower EtOH concentrations could, in extreme cases, interfere with hematoxylin staining

25- Washes are necessary to remove traces of previous solutions and to prepare the sections for the solutions in the subsequent steps. Sections should be fully submerged in liquid at all times.

26- For H&E staining sections thicker than 3-5 μm may require longer exposures to solutions in order to ensure that each reagent fully penetrates the tissue.

27- Inadequate dehydration of the tissue sections will result in slides being milky due to the mixing of water from the EtOH solutions with the xylene of the mounting medium when coverslipping

28- This step helps to displace the EtOH from the tissue sections, ensuring full miscibility with xylene in the mounting medium. If any alcohol remains in the tissue, the eosin may bleed from the tissue section after coverslipping.

29- Avoid using low-grade xylene; industrial or engineering xylenes may contain petroleum products that can interfere with staining.

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FIGURE LEGENDS

Figure 1. Dissection tools.

Figure 2. Schemes for guiding aorta dissection **(A)** and aortic root sectioning (**B)**.

Figure 3. Oil Red O staining and quantification of atherosclerosis burden. **(A)** Example of a freshly dissected intact aorta and ORO-stained aortas with and without atherosclerotic plaques. (**B)** Images illustrating the process for computer-assisted quantification of the area of vessel covered with atherosclerotic plaques in the aortic arch (1, 2) and thoracic aorta (3, 4). 1: total aortic arch area rendered in red; 2: area of aortic arch occupied by plaques rendered in green; 3: total thoracic aorta area rendered in blue; 4: area of thoracic aorta occupied by plaques rendered in yellow. Atherosclerosis burden is typically quantified as ORO-stained area as a percentage of total area.

Figure 4. Overview of the protocol for paraffin embedding and sectioning of aorta or heart (to obtain sections through the aorta and aortic root, respectively); see Methods 3.4 and 3.5.

Figure 5. Examples of hematoxylin & eosin-stained aortic sections. **(A)** Aortic root without atherosclerotic lesions. **(B)** Thoracic aorta without atherosclerotic lesions. **(C)** Aortic root with atherosclerosis plaques. In the image on the right the sinus perimeter is marked in green and the valve perimeter in black, and the atherosclerotic plaques are marked in yellow.

A

To cut the mouse skin and open the ribcage

Straight serrated tips forceps

Straight blunt scissors

B

To remove the aorta

Straight or curved serrated tips fine forceps

Straight sharp fine scissors

C

To remove adventitial fat, open longitudinally the aorta and pin it flat

Micro dissecting spring scissors

Straight fine tips forceps

Minutien pins 0.10 mm

Figure 1

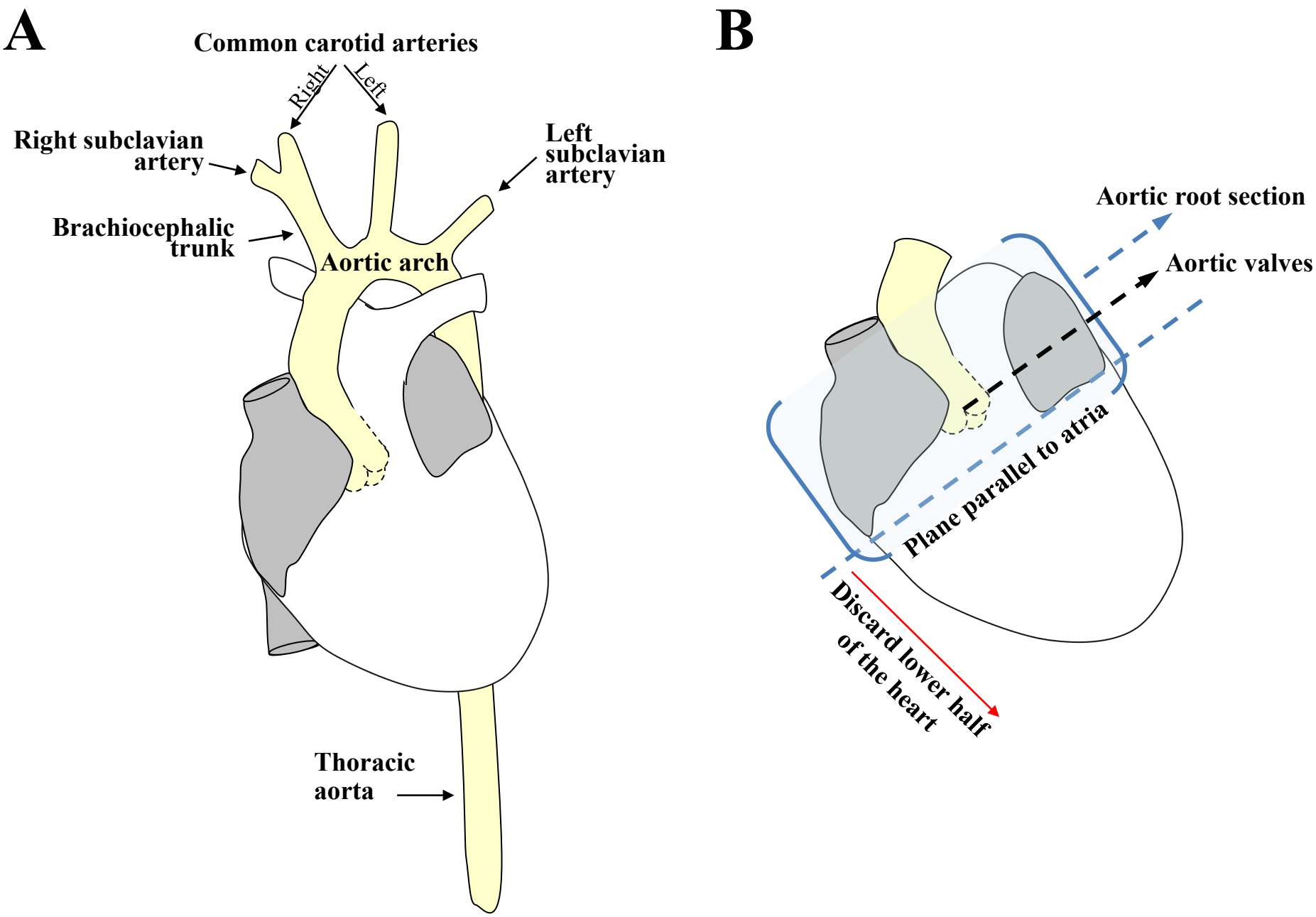
A
Common carotid arteries
Right
Left
Right subclavian artery
Left subclavian artery
Brachiocephalic trunk
Aortic arch
Thoracic aorta
B
Aortic root section
Aortic valves
Plane parallel to atria
Discard lower half of the heart

Figure 2

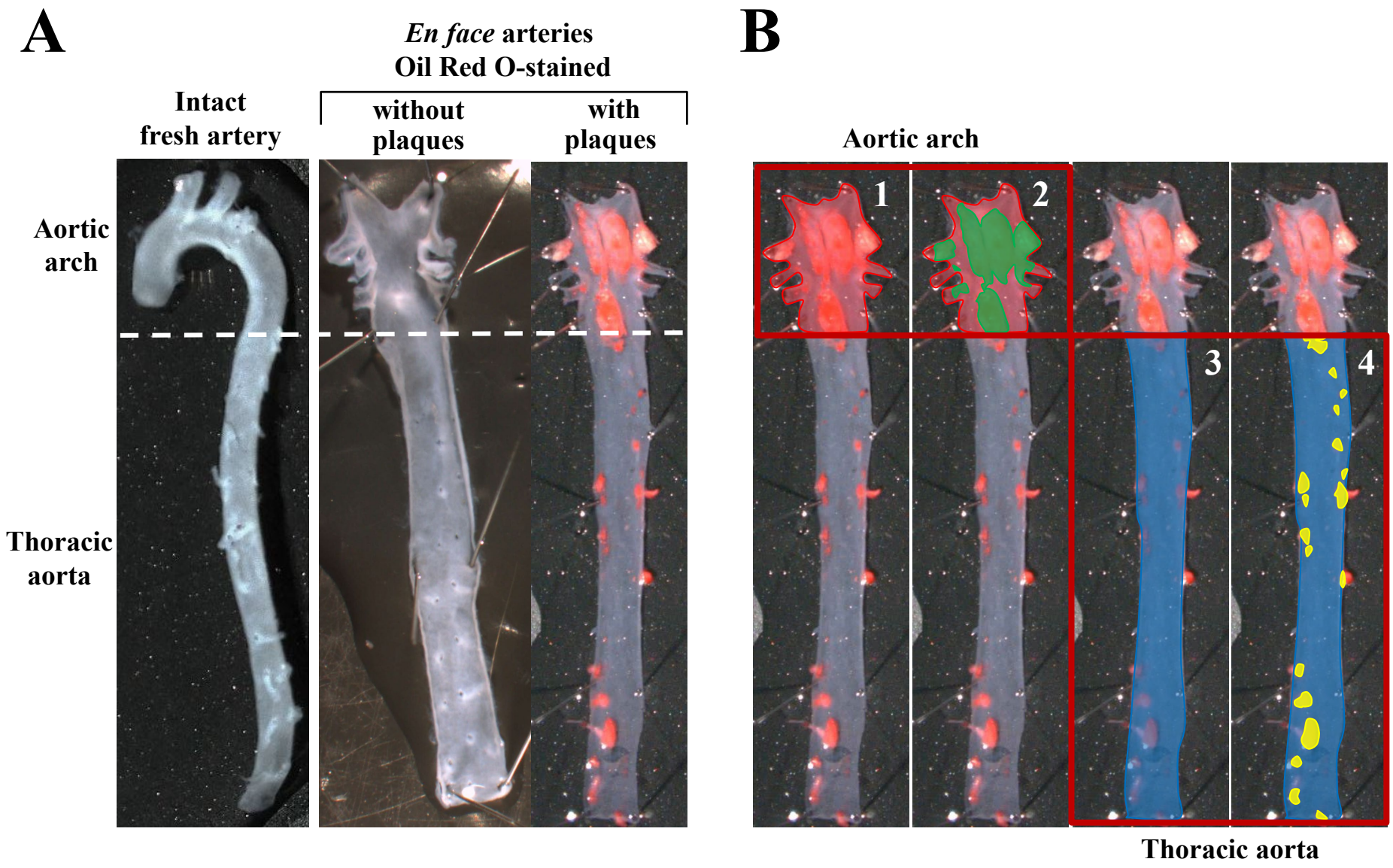
A
En face arteries
Oil Red O-stained
Intact fresh artery
without plaques
with plaques
Aortic arch
Thoracic aorta
B
Aortic arch
1
2
3
4
Thoracic aorta

Figure 3

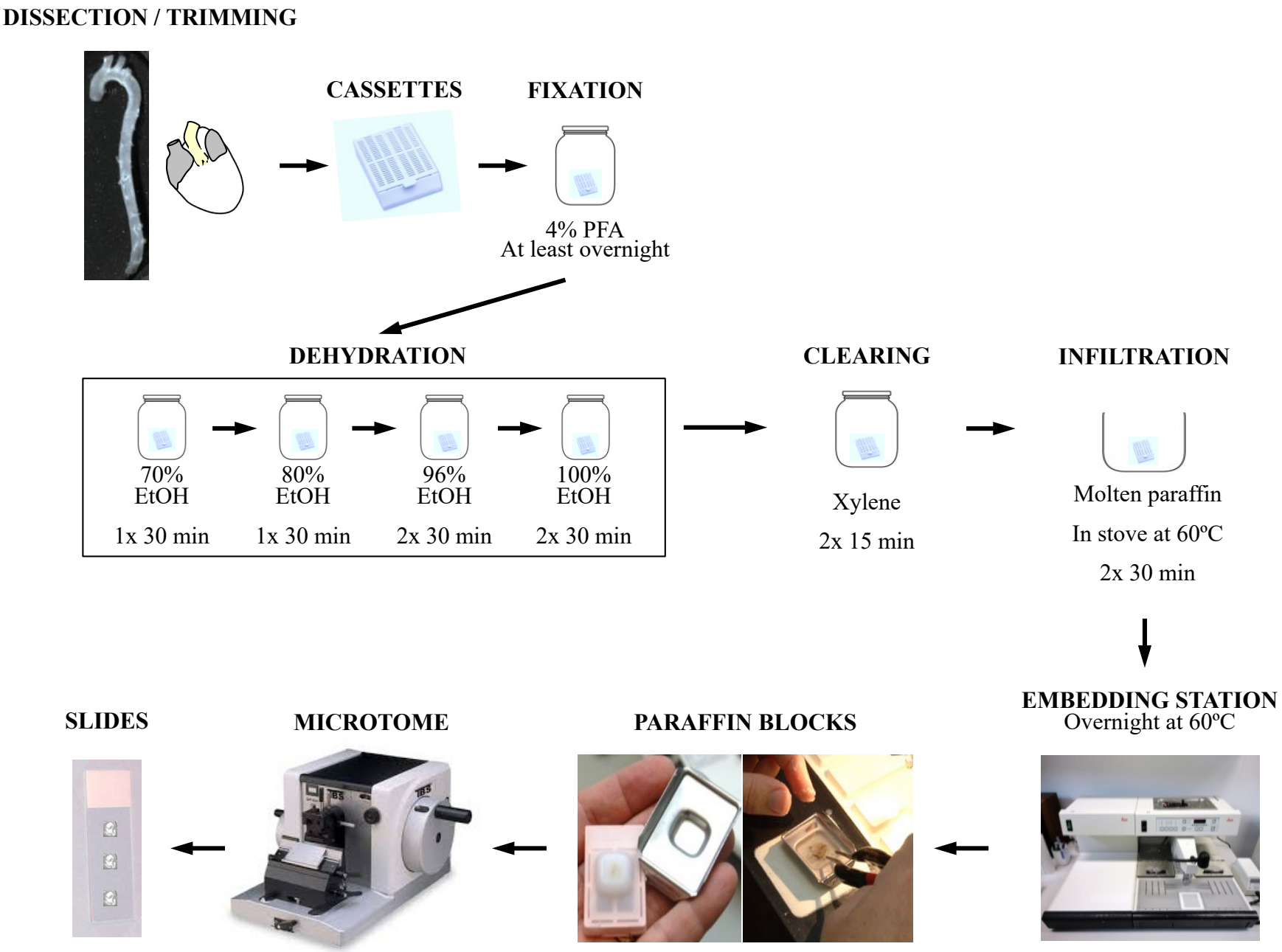
DISSECTION / TRIMMING
CASSETTES
FIXATION
4% PFA
At least overnight
DEHYDRATION
70%
EtOH
1x 30 min
80%
EtOH
1x 30 min
96%
EtOH
2x 30 min
100%
EtOH
2x 30 min
CLEARING
Xylene
2x 15 min
INFILTRATION
Molten paraffin
In stove at 60ºC
2x 30 min
EMBEDDING STATION
Overnight at 60ºC
PARAFFIN BLOCKS
MICROTOME
SLIDES

Figure 4

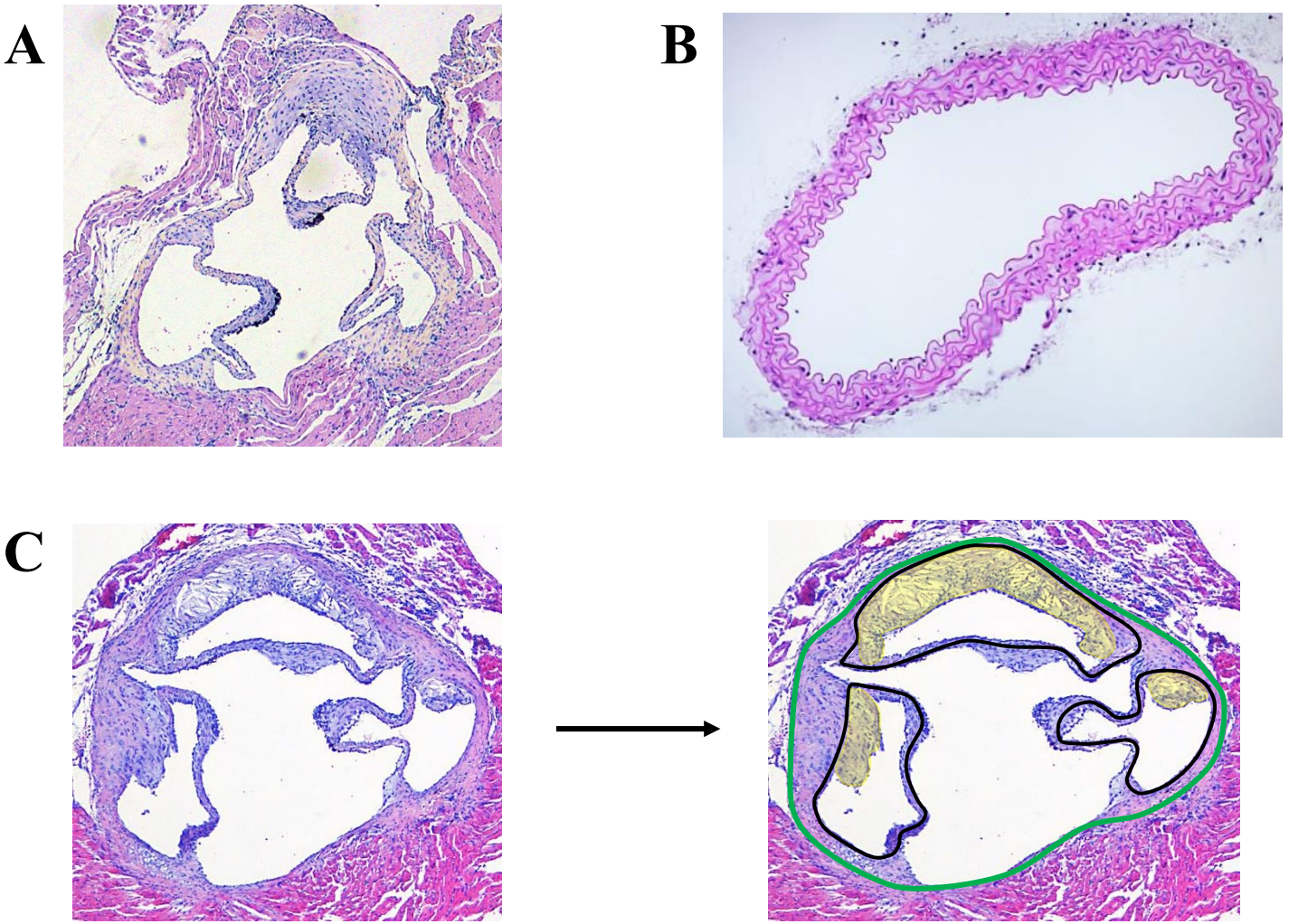

Figure 5